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Transduction Efficiency of Pantropic Retroviral Vectors Is Controlled by the Envelope Plasmid to Vector Plasmid Ratio

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Abstract

Pantropic retroviral vectors pseudotyped with vesicular stomatitis virus envelope G protein (VSV-G) are typically produced by transient transfection of the VSV-G expression plasmid because constitutive expression of VSV-G is cytotoxic. To produce pantropic vectors, the VSV-G expression plasmid and the vector plasmid are cotransfected into a packaging cell line, such as 293-gag-pol. Typically, the ratio of VSV-G plasmid to the vector plasmid ranges from 0.33 to 1.0. However, it is not clear that this range is optimal for vector production. In this study we have systematically examined the effect of the ratio of VSV-G plasmid (pVSV-G) to vector plasmid on vector production. For this, 293-gag-pol stable packaging cells were cotransfected with pVSV-G and an enhanced green fluorescent protein- (EGFP-) expressing retroviral vector plasmid (pLTR-EGFP) by use of lipofectamine. Vector was collected following transfection and used to transduce three target cell lines, namely, 3T3 fibroblasts, telomerase-immortalized human diploid fibroblasts (HDF), and the human hepatoma cell line HuH7. Transduction efficiency was evaluated for vectors produced at different pVSV-G:pLTR-EGFP ratios such that the total amount of plasmid transfected into 293-gag-pol cells was kept constant. Our results indicate that transduction efficiency is greatest when the pVSV-G:pLTR-EGFP ratio is substantially below 1.0. For 3T3 and HDF cells, the maximum transduction efficiency was obtained when a ratio of pVSV-G:pLTR-EGFP ranging from 0.053 to 0.2 was used for transfection. The relative magnitude of this effect was greater for lower transduction efficiencies in control cultures. For HuH7 cells, the beneficial effects were smaller than those observed when HDF or 3T3 cells were used. The difference in transduction efficiency for vector produced under various pVSV-G:pLTR-EGFP ratios was not due to differences in the proliferation of packaging cells or target cells. Further characterization showed that the amount of vector RNA relative to p30^{gag} decreased as the ratio of pVSV-G:pLTR-EGFP increased. These results indicate that transduction efficiency increases with increasing levels of vector RNA as long as a minimally sufficient level of pantropic envelope protein is expressed.

Introduction

In recent years, retroviral and lentiviral vectors pseudo-typed with vesicular stomatitis virus envelope G protein (VSV-G) (pantropic vectors) have been used widely for gene transfer (1–5). Compared to conventional Moloney murine leukemia viral (MoMuLV) vectors that

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bear ecotropic or amphotropic envelope proteins, such pantropic vectors have greater stability during ultracentrifugation to concentrate vector and can infect a wider variety of target cell types (6). It has been shown that VSV-G can bind to phospholipids such as phosphatidylinositol, which is commonly found on the outer leaflet of the cell membrane, instead of to a specific cell-surface protein (2). VSV-G can also interact with phosphatidylserine, which is typically found on the inner leaflet of the membrane. However, a stable packaging cell line constitutively expressing VSV-G cannot be established because constitutive expression of VSV-G is cytotoxic (1,6,7). Instead, an inducible system can be used for production of pantropic retroviral vectors, in which the stable packaging cells express the three components of the vector—the viral structural and replication proteins (Gag/Gag-Pol), the vector RNA, and the envelope protein—at constant levels with respect to each other after induction (6). Alternatively, a transient retroviral vector production system is used in which either (i) packaging cells are tri-transfected with plasmids encoding Gag/Gag-Pol, VSV-G, and vector RNA or (ii) packaging cells that stably express Gag/Gag-Pol are cotransfected with plasmids that express VSV-G and the vector RNA (1). It should be possible to optimize vector production by manipulating the levels of vector RNA and envelope protein. For transient vector production these, in turn, can be adjusted by changing the amounts of the vector plasmid and envelope protein expression plasmid used to transfect the packaging cells.

The effect of changing the ratios of some of these components with respect to each other has been examined in the context of producing amphotropic vector. Although expression of retroviral protein Gag alone is sufficient for viruslike particle (VLP) production, these VLPs are noninfectious because they lack other vector components including vector RNA, envelope glycoprotein, and functional viral enzymes (8,9). An increase in the amount of Gag in an amphotropic MoMuLV vector production system results in a decrease in infectious vector production because it decreases the possibility that vector RNA and sufficient envelope protein are incorporated into the vector (10). It was also found that sufficient envelope protein (Env) in the viral vector is necessary for Env–viral receptor interaction during transduction (11). Increasing envelope protein density could accelerate the rate at which the viral vector specifically binds to the target cell (12). In addition, the amount of Gag-Pol relative to the level of free Gag produced by packaging cells is important to virion formation, stability of the virion RNA dimer, and infectivity (13,14). This is further compounded by the observation that the production of defective viral vectors will also be affected by the stoichiometry of different components. Such defective retroviral particles can decrease transduction efficiency by competing with retroviral vector to bind to the target cell receptors (15).

To our knowledge, the effects of component ratios on the production of pantropic pseudotyped MoMuLV vector have not been previously reported. In this study, the effects of vector RNA and VSV-G expression on the production of VSV-G pseudotyped MoMuLV vector were examined in a 293-based packaging cell line called 293-gag-pol that stably expresses the *gag* and *pol* gene products from MoMuLV. To produce retroviral vectors, 293-gag-pol cells were cotransfected with envelope protein expression plasmid pVSV-G and vector RNA expression plasmid pLTR-EGFP that encodes enhanced green fluorescent protein (EGFP). Different ratios of pVSV-G: pLTR-EGFP were used in the transfections, while the total amount of the two plasmids was held constant. The vectors produced were then used to transduce three different cell lines: 3T3 fibroblasts (3T3), telomerase-immortalized primary human diploid fibroblasts (HDF), and the human hepatoma cell line HuH7 (HuH7). Previously, other groups have reported ratios of pVSV-G:vector plasmid that have varied between 0.33 and 1.0 (16–18). Our results clearly indicate that vector production is optimal when this ratio is far lower, ranging between 0.053 and 0.2. To understand this better, we have examined the amounts of different vector components—

including the capsid protein (p30^{gag}) as a measure of Gag protein levels, VSV-G protein, and vector RNA—from pantropic vectors produced under a variety of conditions.

Materials and Methods

Cell Lines, Cell Culture, and Vector Plasmids

Cell lines 293, 293-gag-pol (1), HDF (19), and 3T3 were maintained at 37 °C in Dulbecco's modified Eagle's medium with high glucose (DMEM) (Mediatech Inc. Cellgro, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Omega Scientific Inc., Tarzana, CA), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Mediatech Inc. Cellgro). The 3T3 cells were made from BALB/C fibroblasts transformed by a standard 3T3 procedure (20). For HDF cells, the medium was also supplemented with 30 µg/mL hygromycin (VWR International Inc., West Chester, PA). HuH7 cells were maintained at 37 °C in Iscove's modified Dulbecco's medium (IMDM) (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT) and 4 mM L-glutamine (Irvine Scientific) (21). pVSV-G encodes the pantropic VSV-G envelope protein. The open reading frame for EGFP was introduced into a derivative of pLXSN with a large multiple cloning site (22), and this plasmid is referred to as pLTR-EGFP in this report. The schematic structures of both plasmids are represented in Figure 1a.

Transfection

Packaging cells were transfected twice before retroviral vector was collected (Figure 1b). Briefly, 293-gag-pol cells were seeded at 2×10^6 cells in a 60-mm dish with 5 mL of DMEM supplemented with 10% heat-inactivated FBS, penicillin, and streptomycin 40–48 h before the first transfection. A total of 6 µg of plasmids pVSV-G and pLTR-EGFP was transfected into cells by use of Lipofectamine-2000 (Invitrogen, Carlsbad, CA). For this, the pVSV-G and pLTR-EGFP plasmids were mixed together in 0.5 mL of serum-free DMEM. Lipofectamine (20 µL) was added to another 0.5 mL of serum-free DMEM. DMEM with lipofectamine was mixed with DMEM containing plasmid DNA and incubated for 20 min at room temperature. After removal of the culture medium from the cells, the preincubated mixture was added to 293-gag-pol cells together with 2 mL of fresh serum-free DMEM. The transfection medium was replaced with 3 mL of fresh DMEM supplemented with 10% FBS 6 h after the first transfection. A second transfection was performed 3 h later to enhance the transfection efficiency by the same procedure as above. Fresh medium was exchanged 14 h after the second transfection. After that, 5 mL of fresh medium was exchanged into culture every 24 h. The supernatants collected at 48 and 72 h were used for the first and second transductions, respectively.

Transduction

To transduce target cells, supernatant from transfected 293-gag-pol cells was used directly without being frozen. Target cells were transduced twice before analysis by flow cytometry for EGFP expression (FL1 channel) (Figure 1b). Briefly, target cells were seeded at 1.5×10^4 cells/cm² in DMEM supplemented with 10% FBS in either 6-well plates or 60 mm dishes 24–36 h before the first transduction. The confluence of the target cells at the time of the first and second transductions was about 10–20% and 20–40%, respectively. The supernatant collected from the transfected packaging cell culture was centrifuged at 1900 RCF for 8 min to remove cell debris and then used for the respective transduction. The proportion of viral vector used for transduction ranged from 0.5% (v/v) to 25% for 3T3 cells, 6.3% to 12.5% for HuH7 cells, and 50% for HDF cells, with the balance fresh medium. The Polybrene concentration used in the transduction was 8 µg/mL. Transduction medium was replaced with fresh medium 10 h after each transduction. The target cells were removed with 1× trypsin-EDTA, suspended in DMEM supplemented with 10% FBS, and

then analyzed by FACScan (Becton Dickinson, Franklin Lakes, NJ) for EGFP-expressing cells 46–50 h after the second transduction. The EGFP-positive cells were judged to be those cells with fluorescence above that seen in the nontransduced cells in the FL1 channel. Because we use three target cell lines and since the titer is dependent upon the target cells infected, we report results in terms of transduction efficiency rather than titer.

To test the effects of defective particles and VSV-G-containing vesicles on transduction efficiency, supernatant containing defective particles plus VSV-G vesicles or VSV-G vesicles alone was harvested 48 h after pVSV-G (4.5 μ g) transfection of 293-gag-pol cells or 293 cells, respectively. Supernatant from 293 cells without transfection was collected as control conditioned medium. Viral vectors were harvested from 293-gag-pol cell culture 48 h after transfection with a ratio of pVSV-G:pLTR-EGFP equal to 0.053. All supernatants were stored at -80°C before transduction. 3T3 cells were used as target cells and seeded in the same way as described above. Before transduction, the viral vector was mixed with different amounts of the respective supernatants and incubated at room temperature for 30 min. Transduction medium was replaced with fresh medium 14 h after transduction. All of the other procedures were the same as described above. Glucose and lactic acid concentrations in the various supernatants described above were measured on a YSI model 2700 biochemistry analyzer (Yellow Springs Instruments, Yellow Springs, OH).

Immunoblot for p30^{gag} and VSV-G Expression

Gag protein p30^{gag} and VSV-G in viral particles were measured by an immunoblot as described by Yap et al. (10). To compare the amount of p30^{gag} and VSV-G in vectors under different conditions, an equal amount of supernatant under different conditions was used. Briefly, 500 μL of supernatant containing viral vectors was pelleted in a microcentrifuge (IEC, Micromax RF) at 14 000 rpm for 1 h. The upper 450 μL of supernatant was carefully removed with a micropipet, and the remaining 50 μL of concentrated vector was washed once with phosphate-buffered saline (PBS). After removal of the upper 450 μL of PBS, the remaining 50 μL of concentrated vector was lysed in lysis buffer (23), mixed with sample buffer (2 $\times$ sample buffer: 4% (w/v) sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 7.0, 24% (v/v) glycerol, 0.01% (w/v) bromphenol blue, and 5 $\mu\text{g}/\text{mL}$ β -mercaptoethanol), boiled, and then loaded into an SDS–polyacrylamide gel electrophoresis (PAGE) gel. The separated protein bands were transferred onto an Immobilon-P membrane (Millipore, Billerica, MA). The membrane was blocked with blocking buffer (0.14 M NaCl, 20 mM Trizma base, pH 7.6, 0.1% Tween 20, 5% BSA, and 0.05% sodium azide) and then hybridized with primary rabbit anti-p30^{gag} antibody (kindly provided by Dr. Rein from the National Cancer Institute or primary mouse anti-VSV-G antibody (anti-P5D4; Sigma–Aldrich, St. Louis, MO). Then the membrane was hybridized with peroxidase-conjugated secondary antibody (donkey anti-rabbit or goat anti-mouse) (Cell Signaling, Beverly, MA) and analyzed by chemiluminescence.

Continuous Sucrose Gradient Ultracentrifugation

Six milliliters of supernatant containing viral vectors, 500 μL in each of 12 tubes, was concentrated and washed by use of a microcentrifuge as described above for immunoblots. The remaining 50 μL of concentrated vector from each tube was pooled and layered over a continuous linear gradient of 10–50% sucrose solution in TNE buffer [25 mM Tris (pH 7.5), 150 mM NaCl, and 5 mM EDTA] containing protease inhibitors and then centrifuged at 29 700 rpm (SW50.1 rotor, Beckman ultracentrifuge) at 4°C for 16 h. Twenty gradient fractions were collected from the bottom to the top of the gradient.

RT-PCR To Examine Expression of Vector RNA

Vector RNA was isolated from vector by use of the QIAamp viral RNA isolation kit (Qiagen, Chatsworth, CA). The vector used for this isolation was recovered from the same volume of supernatant from 293-gag-pol cells transfected with different ratios of pVSV-G:pLTR-EGFP as was used for protein analysis. Then the OneStep RT-PCR kit (Qiagen) was used for reverse transcription–polymerase chain reaction (RT-PCR). The primers used for RT-PCR had the following sequence: 5'-GCC ACA ACC ATG GTG AGC AA-3' and 5'-CTC AGG TAG TGG TTG TCG GG-3'. Finally, DNA was quantified by use of the PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR) and measured with CytoFluorII (Applied Biosystems, Foster City, CA).

Results

We wished to evaluate the effect of changing the ratio of pVSV-G to the vector plasmid in the production of pantropic retroviral vectors. For this we made use of the plasmids shown in Figure 1a and cotransfected them into 293-gag-pol packaging cells that express the MoMuLV Gag and Gag-Pol proteins. In these experiments, the 293-gag-pol cells were grown in 60-mm dishes, and the total amount of plasmids was fixed at 6 μ g for all transfections. It was observed that the transfection efficiency of 293-gag-pol cells under these conditions ranged from 60% to 80% (data not shown). In separate control experiments, we found that a range from 4 to 8 μ g of pLTR-EGFP (in the absence of pVSV-G) provided similar transfection efficiency of 293-gag-pol cells (data not shown), and therefore 6 μ g of total plasmid DNA was used for the transfections. During initial experiments, it was discovered that transduction efficiency of 3T3 cells was greater when the ratio of pVSV-G:pLTR-EGFP transfected into 293-gag-pol cells was substantially lower than the control ratio (1.0), as shown for a typical experiment in Figure 2. We therefore decided to more fully characterize this increase in transduction efficiency and to determine whether it was dependent upon the cell line transduced or the amount and titer of the vector used. We also performed experiments to understand why a lowered ratio of pVSV-G to pLTR-EGFP during transfection results in higher transduction efficiencies.

Different amounts and batches of supernatant containing viral vector were used to obtain a wide range of transduction efficiencies under control conditions with 3T3, HDF, and HuH7 cells. The results for different plasmid ratios are striking. For 3T3 and HDF cells, transduction efficiency was greatest when the pVSV-G: pLTR-EGFP ratio was substantially below 1.0 (Figure 3a,b), specifically between 0.017 or 0.053 and 0.2.

We also found that the maximum benefit due to a lower pVSV-G:pLTR-EGFP ratio on transduction efficiency for 3T3 and HDF cells was greater when transduction efficiency at control conditions (plasmid ratio of 1.0) was lower. This was true both for differences in transduction efficiency due to vector dilution and for differences between replicate batches of vector. For example, if the volume used of control vector produced at a pVSV-G: pLTR-EGFP ratio of 1.0 produced a transduction efficiency of 20% or less, then an equivalent volume of vector produced with a pVSV-G:pLTR-EGFP ratio of 0.053 would give at least 2-fold increase over the control conditions. In contrast, if the volume used of control vector produced a transduction efficiency that was greater than 60%, then the pVSV-G:pLTR-EGFP ratio had a smaller effect on transduction efficiency. This latter effect is to be expected because the control transduction efficiency is already approaching the maximum possible for the system. Thus, adjusting the pVSV-G:pLTR-EGFP ratio is particularly beneficial when low vector titers may be expected. This vector titer effect on the plasmid ratio benefit is clearly illustrated in Figure 3c, which shows that the overall benefit of using a pVSV-G:pLTR-EGFP ratio of 0.053 for 3T3 and HDF cell transductions decreased as the transduction efficiency at control conditions (ratio of 1.0) was increased (e.g., by increasing

the volume of vector supernatant used for transduction). The advantage of using a lower plasmid ratio can also be seen by examining the effect of vector dilution on transduction efficiency (Figure 3d). What can be clearly observed is that vector produced at a pVSV-G:pLTR-EGFP ratio of 0.053 retained greater infectivity at higher dilution than did vector produced at a ratio of 1.0 (control vector).

In contrast to the results for 3T3 and HDF cells, the pVSV-G:pLTR-EGFP ratio had a smaller effect on the transduction efficiency of HuH7 cells (Figure 3e). Also, the maximum benefit did not vary significantly for different transduction efficiencies under control conditions. We believe that the different results are related in part to the fact that we could not profile GFP-expressing HuH7 cells by flow cytometry as well as comparable 3T3 and HDF cells. GFP expression intensity for transduced HuH7 cells is much lower than for 3T3 and HDF cells (Figure 4 and data not shown). This renders it difficult to distinguish between transduced and nontransduced HuH7 cells. We applied an alternative gating method based on forward scatter vs fluorescence channel 1 (EGFP) to the data shown in Figure 4, to distinguish between transduced and nontransduced cells (overlays in Figure 4b,c). However, even with the second gating method, it does not appear that the pVSV-G:pLTR-EGFP ratio affects transduction efficiency in HuH7 cells to the same extent as for 3T3 and HDF cells with the amounts of vector that we tested. Thus, some cell types are less affected than others by the plasmid ratio. However, it should also be noted that although the effect is less for this cell type, there is still as much as a 1.4-fold increase in HuH7 cell transduction for a pVSV-G: pLTR-EGFP ratio ranging from 0.053 to 0.33 versus that for a ratio of 1.0 [$p \leq 0.05$ (two-sided) by the Wilcoxon signed-rank test].

We investigated the mechanisms underlying the benefits of a lower pVSV-G:pLTR-EGFP ratio. We first examined two simple possibilities that might explain our results: first, that a decreased level of pVSV-G could increase the amount of vector produced by the transfected packaging cells, simply by increasing the survival of transfected 293-gag-pol cells; and second, that vector with higher levels of VSV-G may be more deleterious to the transduced cells than vector with lower levels of VSV-G protein. We first evaluated the growth of transfected 293-gag-pol cells under all of the plasmid ratio conditions. Alterations in the plasmid ratio did not affect the growth rate of transfected 293-gag-pol packaging cells over the time course of the two harvests used for the transductions (data not shown). Thus, the observed increase in transduction efficiency upon decreasing the amount of pVSV-G cannot be attributed to increased survival of transfected 293-gag-pol cells. Similarly, vector produced under all of the plasmid ratios used in these experiments did not affect the growth or survival of transduced HDF cells (data not shown).

Next we examined two key components—p30^{gag} as a measure of the Gag protein produced by the 293-gag-pol packaging cells and the vector RNA product from pLTR-EGFP—of vectors produced under different conditions. The amounts of p30^{gag} and vector RNA in viral vectors that were harvested in the first collection are shown in Figure 5. All values were normalized to the values obtained for the control condition in which equal amounts of pVSV-G and pLTR-EGFP were used for transfection. At the highest level of pLTR-EGFP plasmid used (or lowest amount of pVSV-G used), the total amount of retroviral particles released in the supernatant decreased by 50%. This is indicated by a 50% decrease in the amount of p30^{gag} per volume of supernatant (Figure 5b). In general, there was a slight trend of higher p30^{gag} levels in the supernatant for greater pVSV-G:vector plasmid ratios. It is interesting to note that the increase in retrovirus particle production, as measured by p30^{gag} release, at higher pVSV-G:pLTR-EGFP ratios contrasts with the lower transduction efficiencies observed for these ratios.

The level of vector RNA detected in the supernatant did not change much, except for a 50% decrease at a pVSV-G:pLTR-EGFP ratio of 3.0 (Figure 5c). Over the remainder of the pVSV-G titration, there was relatively little variation in the amount of vector RNA. There was even a 30% increase in vector RNA at a pVSV-G:pLTR-EGFP ratio of 0.017. It should be recalled that, at this level of pVSV-G, there was a 50% decrease in the overall amount of released retroviral particles, as indicated by the amount of p30^{gag} in the supernatant (Figure 5b). To summarize, over the wide range of pVSV-G:pLTR-EGFP ratios we have used, there was relatively little change in the amount of vector RNA or in the amount of retroviral particles released.

We evaluated how differences in the stoichiometry of vector RNA:p30^{gag} were related to changes in the ratio of pVSV-G:pLTR-EGFP. The results in Figure 5d show that the observed values correspond well with the theoretical values, such that the RNA:p30^{gag} ratio at the lowest pVSV-G level was twice as high as that for a plasmid ratio of 1.0. The simplest interpretation of these data is that as the amount of VSV-G protein is increased, there is an increase in the fraction of retroviral particles released that do not contain vector RNA.

The amount of pantropic envelope protein VSV-G in the supernatant that was harvested in the first collection increased substantially as the ratio of pVSV-G:pLTR-EGFP used in transfections was increased from 0.017 to 3.0 (Figure 6a). It was not known whether this released pantropic envelope protein was present only in the context of retroviral vector or also in the context of vesicles that bud from the plasma membrane. To examine this, 293 cells (that do not express Gag or Gag-Pol) were transfected with pVSV-G, and the supernatant was harvested and examined for the presence of VSV-G protein. The results showed that VSV-G was present in the supernatant even in the absence of vector production (data not shown). This suggests that VSV-G is present in the supernatant of 293-gag-pol cells not only as a consequence of being a vector component but also by incorporation into other vesicles that bud from cell membranes. To distinguish between these two pools of VSV-G, we examined the distribution of VSV-G in sucrose gradient fractions of supernatants from 293-gag-pol cells transfected at pVSV-G:pLTR-EGFP ratios of 0.053, 0.33, and 3.0. Supernatants were layered on continuous sucrose gradients and then centrifuged at 82000g. Gradient fractions recovered after ultracentrifugation were analyzed by immunoblot. The results (Figure 6b,c) showed that a substantial amount of VSV-G protein is present in vesicles in addition to viral vectors for all conditions tested. We also note that as the ratio of pVSV-G:pLTR-EGFP decreased, there was an increase in vector density, as estimated by the density of the fractions in which Gag protein could be detected (Figure 6c).

Our discovery that the pantropic envelope protein was present in vesicles, as well as in viral vector, led us to test two possibilities: first, whether VSV-G in vesicles would inhibit transduction by vector, and second, whether defective particles, containing Gag and Pol proteins and VSV-G in the envelope, would inhibit transduction by the vector. To produce VSV-G vesicles, we transfected pVSV-G into 293 cells and collected the culture supernatant. To produce defective vector containing VSV-G, we transfected pVSV-G into the 293-gag-pol cells without a cotransfection of the vector RNA plasmid.

The results of experiments with vesicles and defective particles containing VSV-G are shown in Figure 7. Cell supernatants containing VSV-G vesicles alone had no effect on transduction by vector, even when added in 9-fold excess. Also, cell supernatants containing both pseudotyped defective particles and VSV-G vesicles had no effect on transduction by 100 μ L of vector when added at an excess of up to 5-fold. However, supernatant containing defective particles inhibited transduction by about 50% when added in 9-fold excess. Furthermore, supernatant containing defective particles inhibited transduction by 200 μ L of

vector by about $\frac{2}{3}$ when added in 4-fold excess. It should be noted that, under the latter two conditions, no fresh medium was used for the transduction mixture. The concentration of the metabolic byproduct lactic acid was 30.3 ± 0.1 mM in medium from 293-gag-pol cells transfected at a pVSV-G:pLTR-EGFP ratio of 0.053, 30.1 ± 0.7 mM in medium from 293-gag-pol cells transfected with pVSV-G, 25.3 ± 0.7 mM in medium from untransfected 293 cells, 18.4 ± 0.7 mM in medium from transfected 293 cells, and 1.3 ± 0.1 mM in fresh medium. Thus, part of the decrease in transduction efficiency due to supernatant from 293-gag-pol cells transfected with pVSV-G added at 900 μ L (100 μ L of viral vector) and 800 μ L (200 μ L of viral vector) may be due to the lower pH associated with the higher lactic acid levels in the supernatant from 293-gag-pol cells.

Discussion and Conclusions

Many factors can affect the production of retroviral vectors. One of these is the level of expression of the different vector components. Previous studies have shown that the optimal plasmid ratio between viral vector encoding the *lacZ* cDNA (pCLMFG) and packaging vector (gag-pol-env) was 1:1 for the production of amphotropic viral vector in a transient system (24). Similar studies have not been previously reported for pantropic vectors, which have become widely used because they are more stable and can be used to transduce a wider range of cell types, especially primary cells.

We have used 293-gag-pol cells to produce pantropic vectors. This has allowed us to vary the ratio of two components, namely, the vector RNA and the pantropic envelope protein, to test how these components affect transduction efficiency. Our results indicate two things very clearly. First, optimal transduction occurs when the ratio of pVSV-G:vector plasmid ranges from 0.053 to 0.2, which is far below the plasmid ratios that have been reported in the literature. Second, the beneficial effect of using a lower pVSV-G:pLTR-EGFP ratio is even more pronounced when control transduction efficiencies are poor. Put more plainly, reducing the pVSV-G:vector plasmid ratio from 1 to 0.053 can dramatically increase transduction efficiency (e.g., from 20% to 45% or from 10% to 30%). The different relative increases can be explained by the fact that cells are randomly exposed to a vector, which is modeled as a Poisson random variable (25), so that the increase in transduction efficiency with increased titer is not linear.

In our system, the VSV-G protein is present in three different forms including free vesicles, pseudotyped viral vector, and pseudotyped defective particles. All of these forms associate with receptors on different cells with different efficiencies. Altering the ratio of pVSV-G:pLTR-EGFP results in a change in the amounts of the three forms of VSV-G in cell supernatants. We found that high-level expression of VSV-G within transfected packaging cells decreases expression of vector RNA, thus limiting the overall viral vector production.

On the other hand, a decrease of the vector RNA:p30^{gag} ratio can lead to greater production of defective retroviral particles. Under these conditions, the inhibitory effects of such defective particles on the binding of infectious vector to the target cells can become substantial, thus decreasing the overall transduction efficiency. Forestell et al. (15) have demonstrated that defective retroviral particles can interfere with viral vectors during transduction. Our results also indicate that defective particles can decrease transduction efficiency but only when present in large excess (Figure 7) and when no fresh medium is added. In contrast to the case for defective particles, we did not observe any inhibitory effects of VSV-G vesicles.

Our results will help to improve the production of VSV-G pseudotyped retroviral vector in transient systems for both research and clinical use. They also provide useful information for the production of lentiviral vectors, where multiplasmid transient transfection is also used.

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Figure 1. Plasmid structures and experimental procedures. (a) Schematic structure of the plasmids pVSV-G and pLTR-EGFP. (b) Diagram of the transfection and transduction procedures used in this study.

Figure 2.

Typical transduction result when 3T3 cells were used as target cells ($n = 1$). The transduction efficiency was 8.0% under the control condition (with the ratio of pVSV-G:pLTR-EGFP equal to 1.0). All values are normalized to that under the control condition.

Figure 3.

Comparison of transduction efficiency for different batches and amounts of viral vectors among all conditions with (a) 3T3, (b) HDF, and (e) HuH7 target cells. All values are normalized to those under the control condition (plasmid ratio of 1.0). Data points represent the mean $\pm$ SD ($n = 2-5$). The numbers in the legends represent the transduction efficiency range under control conditions for different batches and amounts of vectors. The actual values of the transduction efficiencies are shown in the table in part (f). (c) Effects of transduction efficiency, under control conditions, on the x -fold increase in transduction efficiency when a ratio of pVSV-G:pLTR-EGFP equal to 0.053 was used for transfections, compared to that for a plasmid ratio equal to 1.0. (d) Effect of vector dilution on 3T3 cell transduction efficiency is compared in a typical experiment for vector produced with pVSV-G:pLTR-EGFP ratios of 0.053 and 1.0. (f) Comparison of control transduction efficiency in panels a, b, and e.

Figure 4.

Comparison of EGFP expression between transduced HDF and HuH7 cells. (a) Nontransduced cells. (b) Cells that were transduced with vectors produced under the control condition (pVSV-G:pLTR-EGFP ratio of 1.0). (c) Cells that were transduced with vector produced at a pVSV-G:pLTR-EGFP ratio equal to 0.053. The value indicated in each figure represents the percentage of EGFP-positive cells, as determined by use of gate M1 (horizontal line). For HuH7 cells, the overlays represent another analysis method, in which the EGFP-positive cells were determined by use of a gate in the FSC–FL1 (forward scatter vs fluorescence channel 1) dot plot. The corresponding percentages of EGFP-positive cells are 34.9% and 48.3% for pVSV-G:pLTR-EGFP ratios of 1.0 and 0.053, respectively.

Figure 5.

Gag and vector RNA content in vectors collected 48 h after transfection. (a) p30^{gag} from a representative western blot and vector RNA from a representative RT-PCR gel. (b, c) Relative amounts of p30^{gag} (b) and vector RNA (c). To compare the amounts of different components in vectors produced under different conditions, an equal amount of supernatant was used for all conditions. The amounts of p30^{gag} and vector RNA are normalized to those in vectors produced under the control condition (pVSV-G:pLTR-EGFP ratio equal to 1.0). (d) Observed ratios for RNA:p30^{gag} are shown together with the theoretical ratios calculated from the amount of plasmids used in the transfections. All values are normalized to those under control condition (plasmid ratio of 1.0). The vector RNA:p30^{gag} ratio has been set equal to 1.0 under the control condition for both observed and theoretical values. Data points represent the mean $\pm$ SD ($n = 2$).

Figure 6. Pantropic envelope protein VSV-G is present in both VSV-G vesicles and viral vectors. (a) Relative amounts of VSV-G in supernatant. The amounts are normalized to that in supernatant harvested under the control condition (pVSV-G:pLTR-EGFP ratio equal to 1.0). Data points represent the mean $\pm$ SD ($n = 2$). (b, c) Representative western blots showing the distribution of VSV-G (b) and p30^{gag} (c) in fractions after continuous sucrose gradient ultracentrifugation. Supernatant was harvested from 293-gag-pol cells transfected with a pVSV-G:pLTR-EGFP ratio equal to 0.053, 0.33, or 3.0, as indicated to the left of the gels.

Figure 7.

Effects of VSV-G vesicles and noninfectious particles on transduction efficiency of 3T3 cells. Viral vectors were harvested from 293-gag-pol cell culture 48 h after transfection with a ratio of pVSV-G:pLTR-EGFP equal to 0.053. The vectors were mixed with conditioned medium from 293 cells, VSV-G vesicles from 293 cells transfected with pVSV-G, or defective particles plus VSV-G vesicles from 293-gag-pol cells transfected with pVSV-G, and the mixture was incubated at room temperature for 30 min before transduction. The volumes of viral vector and supplement (conditioned medium or supernatants from transfected 293 cells or 293-gag-pol cells) are shown for each condition. The transduction volume was supplemented to 1.0 mL with fresh medium as necessary. Data points represent the mean $\pm$ SD ($n = 2$).